Benchmarks

Improved Blunt-End Cloning by Replacing *EcoRV* with *Eco32I*

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The restriction endonuclease *EcoRV*, which recognizes and cleaves DNA containing 5'-GATATC-3' sequences, is among the best characterized and most frequently used blunt-end cutting restriction enzymes (2). Its recognition site is present in many cloning vectors currently in use. Compared with that of cohesive ends, blunt-end ligation is generally more difficult because a higher number of background colonies is formed that result from self-ligation of vector molecules (1). We (and others) have found that when using *EcoRV* even "white color" selection often fails so that one ends up with a large number of apparently positive (white) colonies. Here we demonstrate that this problem can be reduced if one uses *Eco32I*, an isoschizomer of *EcoRV* (3), instead.

To compare the cloning efficiencies of *EcoRV* and *Eco32I*, we chose to insert a blunt-ended PCR fragment derived from the human β -catenin gene *CTNNB1* into the GATATC site of vector pZErOTM 2.1 (Invitrogen, Carlsbad, CA, USA). The GATATC site of pZErO 2.1 is an integral part of an open reading frame encoding a lacZ α /ccdB fusion protein. Insertion of a heterologous DNA fragment disrupts this open reading frame and results in both loss of β -galactosidase as well as rescue from the ccdB suicide gene activity. Thus, using pZErO 2.1, self-ligated vector molecules are efficiently selected against, thereby obviating the need for dephosphorylation of vector DNA before blunt-end ligation.

Genomic DNA was isolated from human embryonic kidney-derived 293 cells, and a 675-bp DNA fragment derived from the human β -catenin gene *CTNNB1* was amplified by PCR using primers bcat-fwd1 (5'-ACATTTCCAATCTACTAATG-3') and bcat-rev1 (5'-AAGTTCTGCATCATCTTGATAG-3'). The resulting PCR fragment contains multiple stop codons in all six possible reading frames, ensuring that inactivation of the ccdB suicide gene occurs

Table 1. Cloning Efficiencies

	<i>EcoRV</i>		<i>Eco32I</i>	
	No. ^a of Insert-Positive Colonies	Total No. of Colonies	No. of Insert-Positive Colonies	Total No. of Colonies
V ^b	0	157 $\pm$ 15	0	65 $\pm$ 9
V + F ^c	24 (15%)	157 $\pm$ 15	25 (38%)	66 $\pm$ 4

^aValues are the means of three independent experiments.
^bV, vector
^cF, *CTNNB1* fragment insert

upon insertion of the fragment in either orientation and any reading frame. The PCR product was gel-purified and extracted using ConcertTM Gel Extraction System (Invitrogen) as recommended by the manufacturer. For cloning, the purified PCR fragment was made blunt-ended by treatment with T4 DNA polymerase and column-purified using High Pure PCR Product Isolation kit (both from Roche Applied Science, Basel, Switzerland).

To generate vector suitable for ligation to the *CTNNB1* insert, 500 ng pZErO 2.1 DNA were digested for 60 min at 37°C with 10 U *EcoRV* (Roche Applied Science) or 10 U *Eco32I* (MBI Fermentas, Vilnius, Lithuania) using supplied buffers. The digested vector was purified using the High Pure PCR Product Isolation kit.

A 15- μ L ligation mixture was then set up in a reaction containing 10 ng linearized vector, 10 ng PCR fragment (molar end ratio 1:5), 4 U T4 DNA ligase (Roche Applied Science) in 1 $\times$ ligase buffer. A control reaction lacking PCR fragment was done in parallel. All ligations were incubated at 16°C for 16 h.

One microliter of each ligation mixture was transformed into 50 μ L chemically competent One Shot[®] Top 10 cells (Invitrogen), and the cells were spread on LB agar plates containing 50 μ g/mL kanamycin. To determine the percentage of true recombinant clones, we prepared colony lifts using Biotyne[®] A filters (Pall, East Hills, NY, USA), which were subsequently hybridized to a radioactively labeled probe generated by amplification of an internal 224-bp fragment of *CTNNB1* using primers bcat-fwd2 (5'-GATTTGATGGAGTTGGACATGG-3') and bcat-rev2 (5'-GCTACTTGTCTTGAGTGAAGG-3'). Hybridization-positive colonies

were counted. We did not employ "white color" selection to check for the presence of inserts because in many previous experiments we found that self-ligated vector molecules lacking inserts may give rise to white colonies.

No insert-containing colonies were obtained in the ligation reactions "vector alone" (Table 1). Ligation of the blunt-ended fragment into vector DNA cut by either enzyme, *Eco32I* or *EcoRV*, resulted in the same absolute number of insert-containing clones; however, when *Eco32I*-digested vector was used, a higher proportion of insert-positive clones (38%) was obtained as compared to *EcoRV* (15%). The difference found was highly significant (two-sided *P* value < 0.001; Fisher's exact test). In three independent experiments, the use of vector DNA prepared by restriction with *EcoRV* resulted in the formation of a significantly higher number of background colonies (Table 1).

To investigate the possible reason for the observed difference in the formation of kanamycin-resistant colonies, we checked the integrity of the GATATC cloning site of clones obtained after cutting and simple self-ligation by sequence analysis. Ten clones each from *EcoRV* and *Eco32I* "vector alone" plates were picked, grown, and plasmid DNA was extracted using the High Pure Plasmid DNA Isolation kit. Sequencing reactions were set up following a dye terminator protocol (BigDyeTM; Perkin Elmer, Boston, MA, USA), following the manufacturer's instructions. Sequencing primers were standard M13 forward and reverse primers.

Sequence analysis revealed a frequent single-nucleotide deletion within the vector's cloning site that changed the GATATC motif to GAATC (GATTC in the complementary strand;

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Table 2. Frequent Single-Nucleotide Deletions in Self-Ligated *EcoRV* Sites

<i>EcoRV</i> ^a		<i>Eco32I</i> ^b	
Sample No. Control ^c	Sequencing Result GATATC	Sample No. Control	Sequencing Result GATATC
1	GA ATC	1	GA ATC
2	GAT TC	2	del ^d :f1 ^e
3	GAT TC	3	del:f1
4	GAT TC	4	del:ccd ^f B
5	GAT TC	5	del:M13rev ^g
6	GAT TC	6	del:M13rev
7	GAT TC	7	del:M13rev
8	GAT TC	8	del:M13rev
9	GA ATC	9	del:f1
10	GA ATC	10	del:ccdB

^apZER0 plamid DNA was cut with *EcoRV*, re-ligated, and transformed into chemically competent One Shot Top 10 F' cells. Ten individual recombinant clones were picked, and plasmid DNA was isolated and subjected to DNA sequencing using M13 forward and reverse primers.

^bpZER0 plasmid DNA was cut with *Eco32I* and treated as described earlier.

^cpZER0 plasmid uncut

^ddel, gross deletion extending into : sequence indicated

^ef1, f1 origin of replication

^fccdB, lethal gene ccdB

^gM13rev, M13 reverse priming site

Table 2). The specific (-1) frame shift created by this mutation results in the immediate rescue from the ccdB suicide gene activity, thereby allowing the outgrowth of false-positive colonies. Such single-nucleotide deletions were found in all clones after cutting and self-ligation with *EcoRV*. In contrast, only one of 10 clones derived from *Eco32I*-treated vector displayed this type of single-nucleotide deletion. However, the majority of false-positive clones obtained in these experiments were found to contain large deletions (202 to 849 bp) that extended into the ccdB gene, the M13 reverse sequencing primer binding site, or the f1 origin of replication, thereby removing part or all of the ccdB gene. Since the overall number of such latter clones was found to be significantly lower than false-positive clones derived from *EcoRV*-treated vector, the mutational mechanism leading to single-nucleotide deletions seems to be specifically associated with the use of *EcoRV* and, thus, is mainly responsible for the increased number of background colonies.

We made similar observations with *EcoRV* enzyme aliquots from different lots from the same as well as from different suppliers (not shown). Therefore, we conclude that the observed single-nucleotide deletions are likely the result of an intrinsic activity associated with the *EcoRV* restriction enzyme itself. Cleavage of GATATC sites by *EcoRV* but not *Eco32I* may specifically lead to partially damaged DNA ends that allow self-ligation but prevent the insertion of heterologous blunt-ended fragments. One possible explanation would be that the removal of the terminal base pair is accompanied by a dephosphorylation event affecting the adjacent 5' phosphate group. Removal of both terminal base pairs then may occur but would result in a completely dephosphorylated vector that can no longer re-ligate. Only those molecules that lack one nucleotide and which are partially dephosphorylated would then give rise to background clones. Alternatively, the removal of the terminal base pair may occur only immediately after cleavage and would be caused by a molecule of

EcoRV sticking to one end of the linearized DNA fragment. Whatever the underlying mechanism may be, substitution of *EcoRV* by *Eco32I* should prove helpful in cloning experiments involving GATATC sites in everyday laboratory practice.

REFERENCES

1. Janulaitis, A.A., P.S. Stakenas, M.P. Piatrushite, Iu.B. Bitinaite, and S.I. Klimashauskas. 1984. Specificity of new restriction enzymes and methylases. Unusual modification of cytosine at position 4. Mol. Biol. (Mosk.) 18:115-129.
2. Pingoud, A. and A. Jeltsch. 1997. Recognition and cleavage of DNA by type-II restriction endonucleases. Eur. J. Biochem. 246:1-22.
3. Struhl, K. 1994. Construction of hybrid DNA molecules, p. 3.16.1-3.16.11. In F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, and K. Struhl (Eds.), Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Address correspondence to Dr. Robert Koesters, Division of Molecular Pathology, Department of Pathology, University Hospital of Heidelberg, Im Neuenheimer Feld 220/221, 69120 Heidelberg, Germany. e-mail: r.koesters@dkfz.de

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Robert Koesters and Magnus von Knebel Doeberitz
University Hospital of
Heidelberg
Heidelberg, Germany

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